REMARKS

In the Action, pending claims 1-5 were variously rejected under 35 U.S.C. §112, first paragraph, for assertedly lacking written description and enablement, and under 35 U.S.C. § 103(a) as assertedly obvious in view of Banks et al. (*Peptides*, 17:305-11, 1996) (hereinafter "Banks") further in view of Borges (*Eur. J Pharmacology* 269:243-48, 1994) (herinafter "Borges") and Caro et al. (*Lancet* 348:159-61,) (hereinafter "Caro"). Reconsideration is requested in light of the following remarks.

I. The Subject Matter of the Claims

The subject matter of the claims relates, in general, to methods of modulating the transport of leptin across the blood-brain barrier (BBB).

II. Claim Objections

The Examiner objects to claims 1-4 as assertedly being drawn to non-elected subject matter. The Restriction Requirement of 2/1/05 indicated that Applicant must elect a species "to which the claims shall be restricted if no generic claim is finally held to be allowable," (page 4 of the Restriction). In the response to the Restriction Requirement filed 3/1/05, Applicant's election of the species epinephrine was with traverse, and with reservation of the right to rejoinder of claims upon allowance of generic claims. Applicant submits that the generic claim is still pending and as such the claims are not drawn to non-elected subject matter.

III. Support for Amendment to the Claims

Support for the amendment to the claims is found throughout the specification . For example, page 5, lines 3-12 describes adrenergic agonists contemplated for use in the invention. Support for new claim 77 is found for example, at page 5, lines 3-12, and in Example 7 (page 26), which teaches that isoproterenol and arterenol modulate leptin transport, and in Example 9

(page 28), which shows that cirazoline modulates leptin transport.

IV. Patentability Argument

A. The Rejection of Claims 1-5 Under 35 U.S.C. §112, First Paragraph, for Lack of Enablement Should Properly Be Withdrawn

The Examiner maintains the rejections of claims 1-5 as assertedly not being enabled because, according to the Examiner, Applicant has not enabled transport of leptin across the blood-brain barrier (BBB) using all the compounds recited in the claims, by every route of administration in the claims, and using all said compounds co-administered with all leptins, leptin derivatives and fragments thereof. The Examiner asserts that there are "so many non-working embodiments disclosed in the specification" that the claims are not enabled over the claimed scope.

The claims as amended are drawn to method for transporting leptin across the blood-brain barrier comprising administering adrenergic agonists and exogenous leptin. Applicant has taught that several adrenergic agonists are effective at modulating transport of leptin across the blood brain barrier, including epinephrine, isoproterenol, arterenol and cirazoline (see Examples 7 and 9 of specification). As stated previously, "The enablement requirement is met if the description enables any mode of making and using the invention." Johns Hopkins Univ. v. Cellpro, Inc., 152 F.3d 1342, 47 U.S.P.Q.2D 1705 (Fed. Cir. 1998) (emphasis added). The requirement for enablement is whether or not a person of ordinary skill could make and use the invention and not whether each compound is effective in the method of the invention. Applicant has demonstrated that several members of the class of adrenergic agonists enhance leptin transport, and for members of the class not exemplified herein, has taught methods for measuring the transport of leptin in the presence and absence of these compounds using routine experimentation.

Although not all adrenergic agonists share strong structural similarity, the agents clearly have a functional relationship as evidenced by the understanding of these terms in the art (See Principles of Internal Medicine, Isselbacher et al., Eds. 13th edition, 1994, Ch. 364, submitted with response of October 18, 2005). One of ordinary skill can readily find reference to, description of, and members of the genus of adrenergic agonists in the art (see Borges et al., *infra,* which refers to the genus of adrenergic drugs, including agonists and antagonists, see abstract). Thus, the specification has enabled the genus of compounds as they are commonly identified by the class of compound they represent, and provides sufficient guidance for one of ordinary skill to make and use the invention based on the understanding of the agents in the art.

The Examiner further asserts that because Applicant demonstrated that not all adrenergic agonists enhance leptin transport Applicant has not enabled the methods of the invention. The presence of inoperative embodiments within the scope of a claim does not necessarily render a claim nonenabled. Applicant is not required to demonstrate efficacy of every member of a certain class of compounds, but enable the class such that one of ordinary skill in the art could make and use the invention. Atlas Powder Co. v EI du Pont de Nemours & Co., 750 F2d 1569, 224 USPQ 409 (Fed. Cir. 1984). Applicant provides guidance in the specification as to what compounds are members of the class of agents contemplated, teaches methods for determining if a compound is a modulator of leptin transport, and also teaches that several members of the class of compounds are effective in the methods of the invention, thereby teaching how to carry out the methods of the invention.

With respect to the objection to claim 2, Applicant has amended claim 2 to remove reference to intrathecal administration in order to expedite prosecution.

With respect to the Examiner's previous objection to the enablement of leptin analogs, variants, derivatives and fragments thereof, the Examiner has not specifically re-addressed these objections in the present Action. However, Applicant submits that all of the leptins recited in the claims are enabled in the specification and in the art. For example, U.S. Patents 6,734,160 and 6,471,956 (submitted with the response of June 30, 2005 as Exhibit A) describe and claim leptin fragments and analogs. These fragments are described in terms of where the leptin protein may be truncated or substituted (see U.S. Patent 6,471,956, col. 18 to col. 20). The art also describes methods to determine if a leptin sequence has biological activity, by measuring leptin binding to leptin-specific antibodies, leptin competition assays, and leptin receptor binding assays, all routine experiments regularly performed by one of ordinary skill. Moreover, the specification also teaches, at page 12, line 15 to page 16, line 5, leptin fusion proteins and leptin derivatives having chemical moieties (e.g., PEG). The specification teaches one of ordinary skill in the art how to make a leptin fragment or consensus leptin, teaches that leptins, including fragments and consensus leptins, as contemplated by the invention retain the biological activity of modulating weight or altering metabolism in a host mammal (page 9, lines 11-13), and also teaches methods for measuring transport across the blood-brain barrier (see Example 1). Further, the Examiner states in Section 7 of the Action that leptin fragments and consensus leptins have sufficient support in the specification and prior art.

The Examiner has maintained the objection to claim 1 as an asserted single means claim. The claim does not encompass every method of modulating leptin transport but is specific for modulating leptin transport across the blood brain barrier using a specific category of agents, adrenergic agonists. As such, the assertion that claim 1 is a single means claim directed to all means of modulating leptin transport is incorrect, and should be withdrawn.

Applicant has taught transport of leptin across the blood brain barrier using adrenergic agonists, including epinephrine, isoproterenol, arterenol and cirazoline, and teaches methods of measuring leptin transport with other adrenergic agonists. Additionally, the specification teaches how to make and use a leptin as contemplated by the methods of the invention. Therefore, Applicant has taught a worker of ordinary skill in the art to make and use the methods of the invention, and a person of ordinary skill would only have to use routine experimentation to repeat these methods. As such, the Examiner's rejections under 35 U.S.C. §112, first paragraph, enablement, should be withdrawn.

B. The Rejection of Claims 1-5 Under 35 U.S.C. §112, First Paragraph, Written Description Should Properly Be Withdrawn

The Examiner maintains the rejection to claims 1-5 as allegedly lacking written description, asserting that Applicant has not described all the agents recited in the claims or all leptin variants and fragments encompassed by the claims. Applicant respectfully disagrees.

Claim 1 has been amended to recite methods of modulating leptin transport comprising administration of adrenergic agonists. The claims as amended are drawn to modulating transport of leptin using a single class of compounds all sharing a common functional characteristic. While the species within the class of adrenergic agonists may not all share structural characteristics they are necessarily defined by their functional characteristics, otherwise they would not be members of the particular genus described. See e.g., Principles of Internal Medicine, *supra*. The function of an adrenergic agonist is readily understood in the art and members of the class are widely disclosed in the art and in the specification. The specification sets out a representative list of adrenergic agonists contemplated for use in the methods of the invention (see page 5, lines 3-12) and not an exhaustive list of those compounds because they are readily available in the art. What is conventional or well-known to one of

ordinary skill in the art need not be disclosed in detail in the specification. Hybritech, Inc. v. Monoclonal Antibodies, Inc., 802 F.2d 1367, 231 USPQ 81(Fed Cir. 1996). Applicant's disclosure of representative members of the class of adrenergic agonists, coupled with the general knowledge of the common function of these compounds in the art, demonstrates that a person of ordinary skill would readily understand that Applicant was in possession of the claimed genus of compounds.

The Examiner further asserts that leptin fragments and derivatives are described in the specification, but the specification allegedly does not describe "chemically modified derivatives of leptin, and fragments thereof." Applicant stated previously, and the Examiner acknowledges (see page 7 of the Action), that biologically active leptin fragments, variants, consensus sequences and the like are described in the specification and well-known in the art (See U.S. Patents 6,350,730, 6,309,853, 6,734,160, 6,429,290 and 6,471,956). The specification teaches which leptin sequences are optimal in a leptin fragment polypeptide, which amino acids can be changed to arrive at a consensus sequence and to which amino acids they can be changed (page 10, line 1, to page 12, line 8). Further, the specification at page 12, line 9, to page 16, line 5, describes chemical modifications common in the art and contemplated as chemical modification of leptin, including leptin analogs or fragments of leptin. For example, the specification describes that the leptin useful in the invention may be modified using water soluble polymers, such as polyethylene glycol, dextran, polyvinyl alcohol, and many other chemical moieties well-known in the art (page 13, lines 6-14). U.S. Patents 6,350,730 and 6,429, 290 also describe chemically modified leptins and methods for making.

As stated above, what is well-known to one of ordinary skill in the art need not be disclosed in detail in the specification (Hybritech, Inc., *supra*). Chemical moieties useful for

making modified polypeptides are well-known in the art as are methods for making chemically modified polypeptides. A chemical moiety is readily attached to either a full-length leptin or a fragment or analog of leptin that is described in the specification. Given the description in the specification of leptin, leptin fragments, consensus sequences, and chemical moieties that may be attached to any of the contemplated leptins, and the general skill and knowledge in the art of making any of these leptins, one of ordinary skill in the art would recognize that Applicant was in possession of the invention, including chemically modified leptins and fragments thereof, at the time of filing.

For the reasons set out above, the rejection of claims 1-5 under 35 U.S.C. §112, first paragraph, written description, should be withdrawn.

C. The Rejection of Claims 1-5 Under 35 U.S.C. §103(a), Should Properly Be Withdrawn

The Examiner rejected claims 1-5 under 35 U.S.C. §103(a) as assertedly obvious over Banks (*Peptides* 1996, 17:305-311), in view of Borges (*Eur. J Pharmacol.* 1994, 269:243-48), further in view of Caro (*Lancet* 1996, 348:159-161). The Examiner asserts that because Banks and Caro assertedly teach that leptin requires transport across the BBB, and Borges assertedly teaches that epinephrine increases non-specific permeability of molecules across microvascular cells in vitro, a worker of ordinary skill in the art would be motivated to combine the teachings of Banks, Caro and Borges to arrive at the present invention. The Examiner further asserts that the mechanism of action of the transport is immaterial to the obviousness of the claimed invention (Action, page 8). Applicant respectfully disagrees.

Banks teaches administration of leptin to a mammal to suppress food intake. Banks neither discloses nor suggests administration of epinephrine to increase leptin transport. Caro teaches that leptin levels are correlated with body mass index and obesity, and indicates that

leptin likely crosses the BBB using a saturable transport mechanism. Caro suggests that administration of exogenous leptin would be ineffective because leptin must cross the BBB via a transport mechanism. Caro neither discloses nor suggests the administration of epinephrine in conjunction with leptin.

Borges teaches that administration of 100 nM adrenaline (epinephrine) can increase the permeability of microvascular endothelial cells to impermeable solutes, exemplified by sodium fluorescein bound to albumin. Borges further teaches that at higher concentrations (1000 nM) administration of adrenaline in the in vitro transport model causes a decrease in passive transport, likely due to adrenaline's affinity for β-adrenergic receptors (Page 246, col. 2). Contrary to the Examiner's position (Action , page 9), Borges does teach that transport across the BBB in the in vitro model used is due to increased permeability, stating that "the results obtained in the blood-to-brain transport experiments demonstrate that drugs possessing α-adrenoreceptor agonist properties induce an increase in the monolayer permeability to sodium fluorescein (page 246, col. 2). Borges hypothesizes that the transport is likely due to pinocytosis (page 247, col. 1). Borges neither discloses nor suggests administration of leptin in conjunction with epinephrine to increase leptin transport nor suggests that epinephrine can be used to modulate specific transport mechanisms.

Banks and Caro teach that leptin requires a specific transport mechanism to transport exogenous leptin across the BBB. In contrast, Borges teaches that epinephrine non-specifically increases the permeability of molecules across an artificial BBB model. A person of ordinary skill reading Borges would not reasonably expect that an agent that non-specifically increases BBB permeability would cause specific transport of leptin across the BBB via the specific transport system discussed in Banks and Caro. Therefore, disclosure of a specific transport

system for leptin in Banks and Caro would not motivate a worker of ordinary skill to look to the teaching of Borges, which teaches a non-specific transporter such as epinephrine, to arrive at the methods of the present invention.

Moreover, Applicant submits that the art teaches away from the methods claimed in the present invention. Epinephrine is well-known as a non-specific disruptor of the BBB allowing diffusion or leakage of molecules across the membranes (Sokrab et al., *Acta Neurol Scand.*, 77:387-96, 1988, full article submitted herewith, abstract submitted with Applicant's response of 10/18/05). Administration of epinephrine can lead to permanent neuronal damage and even death in subjects receiving the agent. (Sokrab et al., *supra*). Epinephrine typically acts to increase blood pressure in a subject and is used as a model for hypertension (Sokrab et al., *supra*). Sokrab et al further indicates that opening of the blood brain barrier, e.g. by adrenaline, allows uncontrolled influx of plasma into the interstitial space which modifies the physiological environment of the brain, and also states that that shrunken neurons in areas of extravasation strongly suggest that their pathogenesis is directly related to BBB damage due to permeablization by adrenaline (Sokrab, page 395, col. 2).

One of ordinary skill would have no motivation to administer epinephrine to enhance specific uptake across the BBB, because epinephrine would cause a random non-specific diffusion of all molecules across the BBB as taught by Sokrab et al. Further, the high level of adrenaline shown by Borges to reduce transport across the blood brain barrier is lethal when administered in vivo, and the neuronal damage resulting from opening of the BBB as taught by Sokrab suggest that administration of epinephrine causes non-specific damage in vivo. Thus, the art teaches that different levels of epinephrine may have different effects on BBB transport, and teaches that permeabilization of the BBB by adrenaline may be detrimental to brain cells.

The mechanism of transport is then, in fact, relevant to the claimed invention. One of ordinary skill would reasonably expect, based on the teachings in the art, that administration of epinephrine would disrupt the BBB to such an extent that any molecule would diffuse across the BBB membrane, potentially mediating harmful influx of proteins across the BBB, thereby negating the specific effect desired by the administration of epinephrine and leptin as disclosed herein. On the other hand, Caro discloses that administration of exogenous leptin would be ineffective due to saturation of the specific transport mechanism. As such, one of ordinary skill in the art reading Borges would not look to the disclosures of Banks or Caro because the non-specific effects of epinephrine in the BBB transport mechanism of Borges would not motivate a person of skill to look to a disclosure describing a specific BBB transport mechanism as in Banks or Caro.

Applicants have discovered, in spite of the teachings in the art, that administration of epinephrine enhances specific uptake of exogenous leptin across the BBB (see Example 3, pages 20-21 and Table 3). There is certainly no indication in Borges, or Banks and Caro, that epinephrine may enhance specific transport of any molecule across the BBB. Given this lack of teaching in the art, there would be no reasonable expectation of success at obtaining the specific transport of leptin as taught in Caro by administering epinephrine according to the teaching of Borges. One of ordinary skill would have no motivation to combine the teachings and arrive at the present invention based on the disclosures of Banks, Borges and Caro. Therefore, the rejection of claims 1-5 under 35 U.S.C. §103(a) should be withdrawn.

V. Conclusion

Applicants submit that the application is now in condition for allowance and respectfully request notice of the same.

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Respectfully submitted,

By _____

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Adrenaline-induced hypertension: morphological consequences of the blood-brain barrier disturbance

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ABSTRACT - Acute hypertension may transiently open the blood-brain barrier (BBB). To determine whether such temporary exposure of the brain parenchyma to plasma constituents may lead to permanent morphological alterations, acute hypertension was induced by i.v. adrenaline in conscious rates given Evan's blue and horseradish peroxidase as tracers. The brain were perfused *in situ* 24 h later: 17 of 21 brains showed multifocal sites of extravasation of the tracers and of endogenous plasma albumin, fibrinogen and fibronectin identified by immunohistochemistry. The proteins spread locally in the parenchyma and were taken up by neurons. Within the leaking sites in the cortex, hippocampus, thalamus and basal ganglia some shrunken and grossly distorted acidophilic neurons were present. Focal areas of sponginess were observed in the subpial and subependymal zones. Thus, a transient opening of the BBB may lead to neuronal damage.

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The blood-brain barrier (BBB) regulates the movement of substances between the plasma and the extracellular fluid in the brain. This important function can easily be influenced in many pathological conditions. For instance, acute rise in the arterial blood pressure can produce transient, focal opening of the BBB in experimental animals (1, 2). This can lead to indiscriminate influx of various biologically active plasma substances into the brain and alter the composition of the extracellular fluid and hence upset the normal physiological microenvironment around the neural cells.

Comprehensive knowledge of the consequences of the opening of the BBB in acute hypertension is lacking. There are some findings indicating that neurons are affected very early after the onset of the BBB opening. For instance, animals with focal barrier opening in acute hypertension occasionally showed spontaneous abnormal electroencephalographic recording over the leaking areas (1), and convulsions may occur in conscious rats (3). Furthermore, an increased glucose utilization has been observed in the acute stage after a hypertensive opening of the BBB (4).

Previous studies have shown that the function of the BBB rapidly returns to normal after a transient opening induced by acute elevation of the arterial blood pressure (5, 6). The question

therefore arises if such a transient opening of the BBB is associated with more permanent cell injury detectable as structural changes in brain. To our knowledge there is no documentation of definite histopathological changes in neurons or glial cells associated with BBB opening in acute hypertension. Earlier studies on acute hypertension have used short survival times and predominantly been concerned with the tracer passage over the endothelial cell membrane. A few earlier studies have reported uptake of tracers in the neurons and the glial cells in the acute stage, i.e. from minutes to a few hours (1, 7, 8, 9). This work was therefore initiated to study possible cellular changes in the brain of conscious unrestrained rats 24 h after an episode of acute hypertension induced by adrenaline. Adrenaline was chosen because it opens BBB more efficiently than other vasoactive substances in conscious rats (10).

Material and methods

Twenty-one Sprague Dawley rats (Møllegaard Breedig Center, Copenhagen, Denmark) weighing 220–470 g were used. The rats had free access to drinking water and food pellets until the time of operation.

For macroscopical identification of areas with BBB opening all the rats were given Evans blue, which *in vivo* binds to serum albumin. The extravasated dye/albumin complex (EBA) can easily be identified by the naked eye, in the perfused brain. In addition, 3 rats received horseradish peroxidase (HRP). In rats not receiving HRP, the extravasated endogenous plasma proteins albumin, fibronogen, and fibronectin were demonstrated by an immunohistochemical method (11–13). Parallel sections were stained with hematoxylin – eosin or acid fuchsin/celestine blue. By using parallel sections close comparison could be made between protein extravasation and cellular alterations.

Operative procedure

A femoral artery and vein were cannulated by polyethylene tubing (Portex PE 50) Under intraperitoneal methohexital (Brietal, Eli Lilly, Sweden AB) and the catheters were exteriorized on the back of the neck. The animals were allowed to recover from the anaesthesia for at least one hour. Then 270 IU/kg heparin was given i.v. and the artery catheter was connected to a pressure transducer. A graphic recording of the mean arterial pressure (MAP) was obtained. The $PaCo_2$, PaO_2 and pH were measured with a microelectrode analyser (IL 413, Instrumentation Laboratory, USA) operating at 37°C.

Evan's blue was given in a dose of 3 ml/kg with 2% solution in saline. 20 min later adrenaline bitartrate, diluted in physiological saline to a concentration of 100 µg/ml, was given i.v. either as a bolus (10 µg/kg) or as infusion (6 µg/kg/min for 10 min) using a syring pump (Sage Instruments, 351, Orion Research Inc., Mass 2139, USA). HRP type VI (Sigma Chemical Co., St. Louis, MO 63178, USA) was slowly given i.v. (30 mg disolved in 0.6 ml saline) to 3 rats 5 min after the start of adrenaline infusion. The drugs were administered simultaneously by a catheter with 2 peripheral tubes and a short common entry. The adrenaline infusion lasted about 2 min after the completed HRP injection.

Fixation and histologic procedure

Demonstration of endogenous proteins and cellular changes. After a 24 h survival period thoracotomy was performed under intraperitoneal methohexital anaesthesia and a cannula was introduced into the ascending aorta through the left ventricle. Heparin, 270 IU/kg was injected into the heart prior to the insertion of the cannula. The descending aorta was then clamped. After an initial flush with physiological saline, 4% formaldehyde in 0.1 mol/l phosphate buffer at pH 7.4 and 37°C was perfused at the pressure of 150 cm H_2O for 15 min.

The perfused animals were kept in the refrigerator until the next day when the brains were removed and immersed in buffered formaldehyde. The brains were cut into 2 mm thick coronal slices and the areas of EBA extravasation were recorded. The tissue slices were embedded in paraffin. Five µm thick sections were cut and stained with hematoxylin/eosin or double stained with 1% acid fuchsin and celestine blue. Parallel sections were exposed to rabbit antisera to rat albumin (Capel Worthington Biochemi-

cals, Malveren, PA 19355, USA), human fibrinogen and human fibronectin (Dakopatts A/S, Glostrup, Denmark). Selected sections were also incubated with antiserum to glial fibrillary acidic protein (GFAP, Dakopatts A/S). The bound antibodies were visualized using the avidin-biotin-peroxidase (Vectastain, Burlingame, CA 94010, USA) with diaminobenzidine as the chromogen. More detailed information about this technique has been presented in previous papers (11–13).

HRP tracing. The procedure was the same as above until the flushing with physiological saline was done. Then 400 ml of 1.25% glutaraldehyde-1% paraformaldehyde mixture in 0.1 mol/l phosphate buffer at pH 7.4 and room temperature was perfused (14). Half the quantity was infused as rapidly as possible while the other half was adjusted at a lower rate to make a total infusion time of 30 min. This was immediately followed by perfusion of 10% sucrose in 0.1 mol/l phosphate buffer at 4°C (15) in quantities and rates similar to the fixative. The brains were then immediately taken out and stored in phosphate buffer-sucrose at 4°C. Within one week the brains were cut into 40 µm thick frozen sections at levels showing EBA extravasation and collected in a container of phosphate buffer and kept at 4°C for 2 h. The sections were then incubated in a medium containing sodium nitroprusside (Sigma Chemical Co., St. Louis, Mo 63178, USA) and tetramethylbenzidine (TMB). The enzymatic reaction was initiated by adding 0.3% hydrogen peroxide. The tissue slices were then transferred to a stabilization solution containing sodium nitroprusside in absolute alcohol. Finally they were washed, mounted on glass slides and left to dry over night at room temperature (16, 17).

RESULTS

Physiological findings

The values of the MAP, $PaCO_2$, PaO_2 and pH before adrenaline infusion are given in Table 1. All the animals responded to adrenaline by a rapid rise of blood pressure (Table 1). After a bolus injection, the pressure reached its maximum level within a few seconds and remained high for about 30 s, whereas with infusion the pressure reached the maximum in 10–15 s, remained at the peak value for 3–4 min and then decreased gradually but remained higher than initial level throughout the infusion. During the HRP injection a transient lowering of the blood pressure occurred. On terminating the adrenaline infusion the pressure came rapidly down to 10–15 mmHg below the initial pressure and then returned to the preinjection level within a few minutes.

Blood-brain barrier changes

Evan's blue. Macroscopically, EBA extravasation was seen outside the areas normally devoid of BBB in 17 of the 21 animals including all the 3 rats which had received HRP. The blue areas were multifocal and often rounded in configuration with indistinct outline. They occupied most frequently the parasagittal areas of the pareital and occipital lobes over the convexity of the brain. Small blue areas were often detected also in the lateral parts of the cerebellar hemispheres and occasionally in the hippocampus and the basal ganglia.

Table 1
The mean arterial pressure (MAP) before and during adrenaline administration by bolus injection or pump infusion. $PaCO_2$, PaO_2 and pH values before induction of hypertension.

Experimental group	No. of animals	Initial MAP (mmHg)	Maximum MAP (mmHg)	$PaCO_2$ (kPa)	PaO_2 (kPa)	pH
Adrenaline bolus injection 10 mg/kg	10	119 ± 4	190 ± 3	5.5 ± 0.2	12.2 ± 0.6	7.39 ± 0.02
Adrenaline infusion 6 mg/kg/min	11	118 ± 4	181 ± 7	5.5 ± 0.1	12.1 ± 0.5	7.38 ± 0.01

Mean values ± S.E.M.

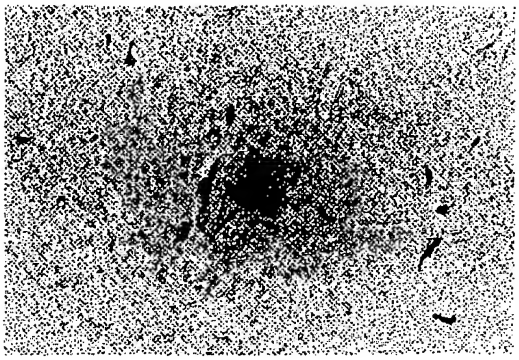

Fig. 1. Focal BBB lession as indicated by extravasated HRP. The reaction product is present in "cellular" profiles presumably neurons as well as in pericytes of vessel walls. TMB histotechnique (× 165).

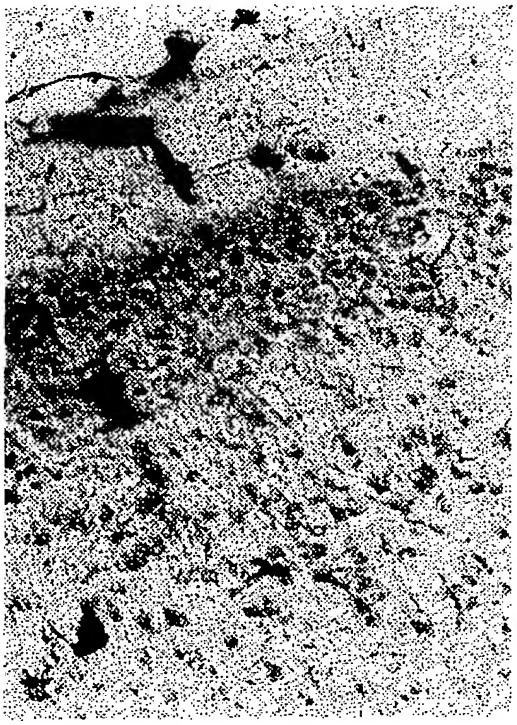

Fig. 2. Extensive granular labelling of cells, obviously nerve cell bodies, in the cerebral cortex of a BBB lesion. The cellular uptake of HRP is so marked that the layers of the cerebral cortex can easily be seen (TMB histotechnique, × 330).

Horseradish peroxidase. Light microscopically, HRP extravasation was detected in all the areas showing macroscopical EBA extravasation including the regions normally lacking BBB. The areas with HRP leakage were, however, in general larger and more frequent than those detected with EBA.

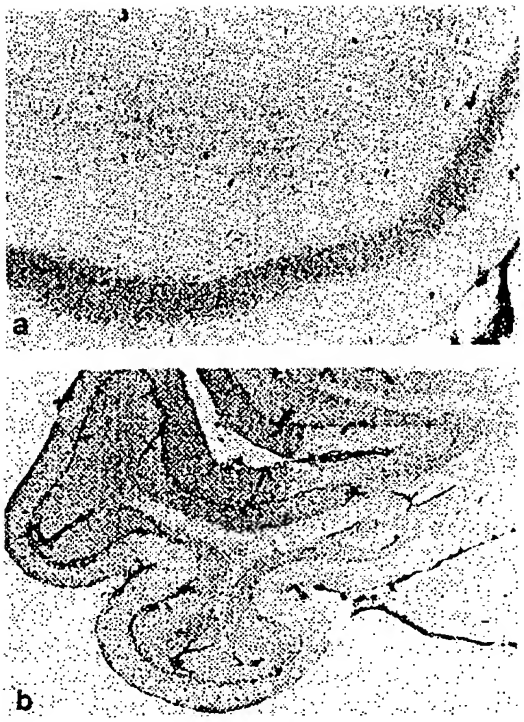

Fig. 3. Dense HRP product in the cytoplasm of neurons in the dentate gyrus of the hippocampus (a) and in all cortical layers of the paraflocculus (b). Note the marked HRP stained Purkinje cell layer (TMB histotechnique).

The smallest lessions consisted in rounded exudates of densely packed, dark brown particles (Fig. 1). In such very dense areas it was impossible to see if the HRP had been taken up into the cells or not.

In some areas of the cerebral cortex particularly in the parieto-occipital region a very marked uptake of HRP into the neurons was seen (Fig. 2), making the various cortical cell layers stand out prominently. The resolution of the technique does not allow to determine precisely if the neuronal uptake was of granular or diffuse variety (*cf.* 18, 19) but as shown in Fig. 2 granular uptake was apparent at least in some of the lesions. Similar marked uptake into neurons occurred in the stratum pyramidale as well as in

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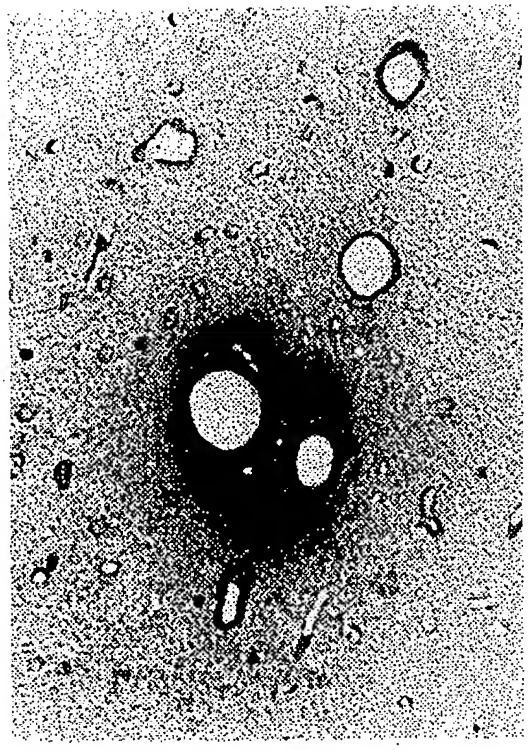


Fig. 4. An arteriole in the fused hippocampal fissue shows strong albumin immunoreactivity in its wall. The immunoreactive material spreads into the surrounding neurophil to a short distance (Antialbumin & hematoxylin, × 330).

the fascia dentata of the hippocampus (Fig. 3a) and the Purkinje cells of the cerebellum (Fig. 3b).

Spread of HRP into axons was seen in the white matter close to the areas of exudation in the cerebral cortex. Besides, spread into dendrites was suggested by the presence of labelled dendritic profiles in the molecular layer of the cerebellum probably originating from Purkinje cells.

Immunohistochemistry. Areas normally devoid of BBB regularly showed endogenous proteins immunoreactivity which served as positive controls for the reaction. The distribution of the immunoreactivity corresponded well to the pattern of EBA extravasation. In general, albumin immunoreactivity was more intense and widely distributed when compared with that of fibrinogen and fibronectin. The distribution of

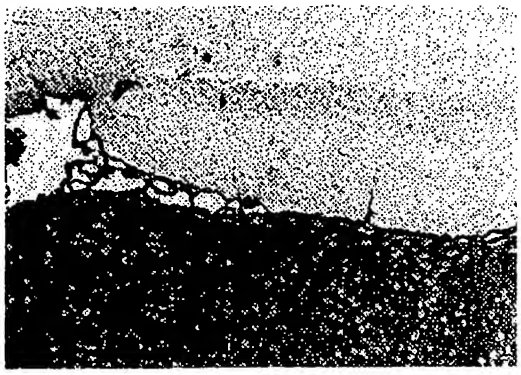
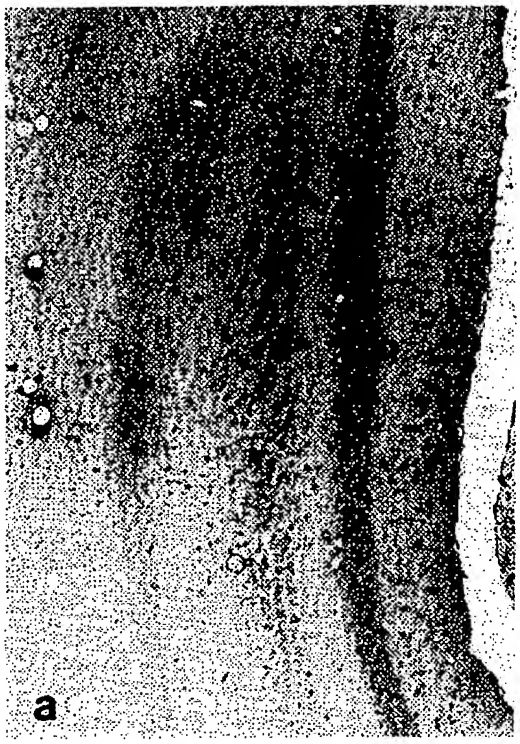


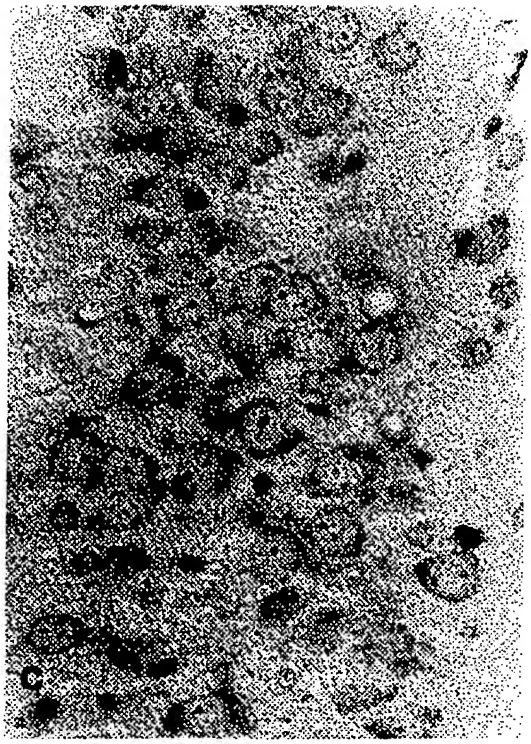
Fig. 5. From an area of a large focal extravasation in the dorsal and lateral part of the thalamus. Albumin immunoreactivity is seen diffusely in the neutrophil and neurons. Note that some of the adjacent neurons are entirely devoid of the immunoreactivity (Antialbumin & hematoxylin, × 100).

albumin immunoreactivity was more extensive than macroscopically observed EBA extravasation.

A large number of arterioles and capillaries showed marked uptake of immunoreactive material in their walls and occasionally in the immediate perivascular zone (Fig. 4). The leakage sites appeared as multiple brown foci of immunoreactive material. Fig. 5 shows such a focus with albumin immunoreactivity in the neuropil and in neurons. In the subpial and subependymal zones immunoreactivity was present as a distinct and extensive band.

There were two different patterns of immunoreactivity which were most distinct with antialbumin serum. Some of the neurons and glial cells in areas of extravasation took up the immunoreactive material in their cytoplasm, and it was also visible in the process of some neurons. Many of these albumin-positive neurons appeared structurally normal with regular nucleus and normal shape (Figs. 6a, b). However, some of the albumin-labelled neurons within the focal leakage site looked shrunken and distorted. Adjacent to stained neurons albumin-negative neurons were also present (Fig. 6c). Astrocytic glial reaction, demonstrated by GFAP immunostaining, occurred in some of the regions with plasma protein extravasation (Figs. 7a, b). The second pattern was a perineuronal aggregation of





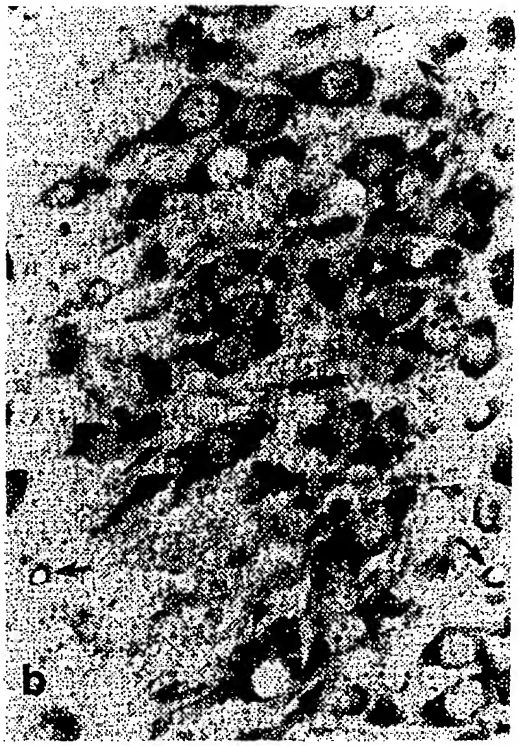


Fig. 6a. Low power picture of the hippocampus shows albumin immunoreactivity in the upper-medial part (close to the hilus) whereas more basally and laterally the immunoreactivity disappears (Anti-albumin & hematoxylin, × 66).

Figs. 6b, c. Higher magnification of the same area as in Fig. 6a. The cytoplasm of the pyramidal neurons in the hilar part of the CA3 regin stains strongly with anti-rat-albumin serum indicating uptake (b), whereas CA3 pyramidal neurons father away from the hilus are negative (c). Note the patent blood vessels (arrows) and the normal appearance (i.e. finely dispersed nuclear chromatin) of the neurons which have taken up albumin (Anti-albumin & hematoxylin, × 270).

immunoreactive material forming a rim around the nerve cell with a clear cytoplasm and normal cellular appearance (Fig. 8). This was seen particularly in some areas in the cerebral cortex and the hippocampus, and it could appear also quite remote from the centres of leakage sites.

Histopathology. A petechial haemorrhage was seen in the caudo-putamen in one brain and a subarachnoid haemorrhage in another.

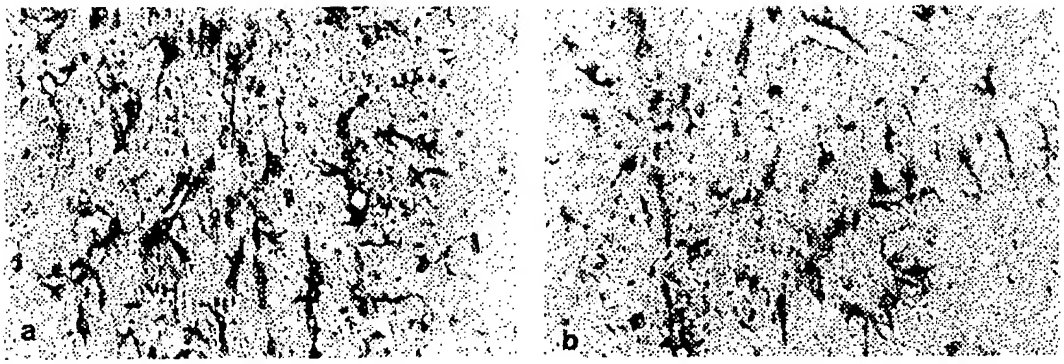


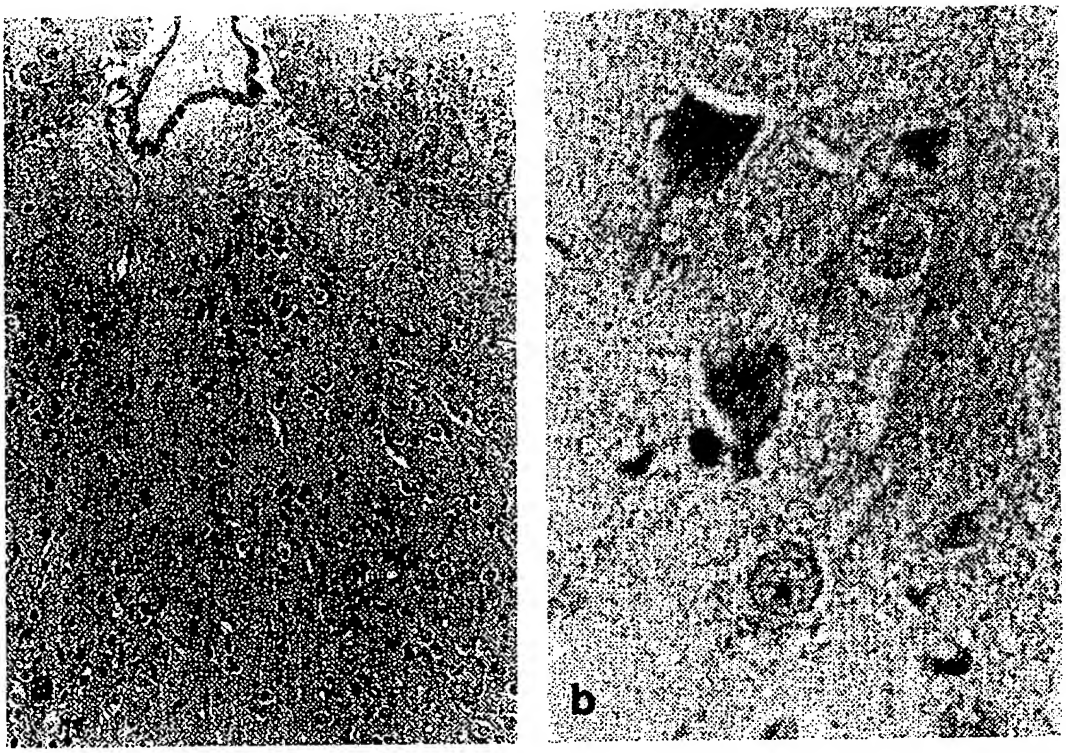
Fig. 7a, b. The GFAP-positive network (astrocytes and their processes) appear more extensive in the cerebral white matter which was positive also for extravasated albumin (a) than on the contralateral side without leakage (b). This implies that astrocytic glial reaction has occurred already at 24 h after BBB opening (Hematoxylin, × 390).

Fig. 8. In the diffusely stained cortex, two neurons and dendrites of one of them are lined by albumin immunoreactivity whereas the cytoplasm remains unstained as do the other neurons nearby. This pattern suggests that albumin gathers around and possibly even attaches to the surface of a specific population of neurons (Antialbumin & hematoxylin, × 550

Focal areas of sponginess were present in the subpial and subependymal zones. Perivascular sponginess was evident around many vessels in areas with extravasation of immunoreactive material. Within leakage sites in the cortex, hippocampus, thalamus and basal ganglia some focally distributed neurons were shrunken and grossly dissorted. Their condensed cytoplasm were characteristically stained brigt red with acid fuchsin (Fig. 9, 10).

Discussion

Various forms of labelled albumin are extensively used in qualitative and quantitative studies concerning the BBB and brain edema. In the immunocytochemical study we chosed to visualize the endogenous proteins albumin, fibrinogen and fibronectin. The evaluation of the findings was based on our immunohistochemical study on



Figs. 9a, b Numerous shrunken dark neurons in dorsal thalamus stained red with acid fuchsin (a). The cytoplasm also stained positively for albumin (× 165); b. Higher magnification (× 790) of the neurons. The sponginess of the neurophil suggests vasogenic edema verified by immunoreactivity for plasma proteins. Acid fuchsin/celestine blue.

the normal distribution of these compounds in the rat brain (13). Recently, fibrinogen was shown to leak in cerebral lesions observed in stroke-prone spontaneously hypertensive rats (11). Growing interest has recently developed in studying fibronectin in the normal and pathological conditions of the nervous system (20, 21, 22). In previous studies (12, 13) we have demonstrated that the soluble form of fibronectin in the blood does not pass the BBB and is a potentially useful indicator of barrier dysfunction.

In the present study all the three tracer techniques used, i.e. EBA, HRP and immunohistochemistry, confirmed that acute hypertension induced by adrenaline in conscious rats produces BBB opening and extravasation of circulating proteins (3, 10). Besides, it was shown that endogenous plasma proteins visualized immunohistochemically can be effectively used as indicators of BBB opening 24 h after a short lasting acute hypertensive episode.

It has previously been shown with EBA and HRP methods that proteins can be observed in nerve cell bodies within minutes to a few hours in areas with BBB dysfunction induced by acute hypertension (1, 7, 8, 9, 23). The present study, based on immunostaining for endogenous proteins, showed that the neurons were definitely influenced in the areas of extravasation 24 h after the hypertensive episode. Signs of albumin immunoreactivity were present to a very large extent in the neuronal perikarya. There were also indications that these proteins had entered axons. Most likely proteins will distribute in the entire neurons by means of axonal and dendritic transports in the same way as has been described for HRP in, for instance, a focal cold injury of the brain (18, 19). Why certain neurons take up

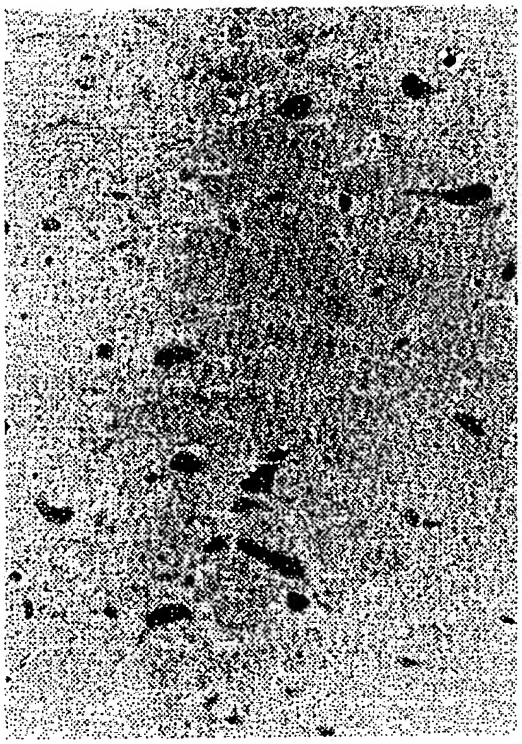


Fig. 10. Several condensed acidophilic neurons which also showed albumin immunoreactivity, are seen focally among normally looking ones in the hippocampal CA1 (Acid fuchsin/celestine blue, × 300).

albumin and HRP whereas others are only "frosted" as if the proteins were only attached to the cell surface remains an open question. Many of neurons with cytoplasm positive for albumin or HRP appeared otherwise intact. Protein uptake in lysosomal organelles by living neurons is a well known phenomenon (cf. 17) and this mechanism is likely to occur also in the present experimental model though the resolution in the light microscopical sections does not allow more precise analysis.

The dense albumin immunostaining seen around arterioles may represent plasma proteins drained from a focus of BBB leakage through the paravascular fluid circulation previously described between the extracellular and the subarchnoid spaces (24, 25).

The neurons staining brilliant red with acid fuchsin were seen in areas of extravasation. Such shrunken acidophilic neurons were previously described in experimental hypoglycemia (26) and status epilepticus (27). A sequential study of hypoglycemia indicated that they are dying cells and that they eventually will disappear from the brain (28). The frequent anatomical localization of the acidophilic shrunken neurons in our study in areas of extravasation strongly suggest that their pathogenesis is directly related to BBB damage. Opening of the BBB allows uncontrolled influx of plasma into the interstitial space which modifies the physiological microenvironment of the brain and might bring biologically active factors in contact with the neurons. Another possibility is that the metabolic effect of the infused adrenaline on the brain could be responsible for the neuronal changes. The catecholamines noradrenaline and adrenaline significantly increase the cerebral oxygen and glucose sonsumption when the BBB is opened (29, 30). In any case, our results are of presumptive clinical relevance since the catecholamine content in the blood is likely to be high in conditions associated with acute hypertensive crisis in man such as eclampsia and pheochromocytoma.

Thus, our results show that neurons may be injured by a shortlasting opening of the BBB. To further elucidate the pathogenetic mechanism and to establish the extent of permanant tissue alterations after acute hypertensive episodes this study will be extended by using a non-pharmacological experimental model of acute hypertension with longer survival periods.

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